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Application of thiophilic membranes for the purification of monoclonal antibodies from cell culture media

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Abstract

The application of thiophilic membranes for the purification of monoclonal antibodies from hybridoma culture media was studied. Affinity filtrations were performed with membrane stacks and also in a cross flow module with a spiral filtration channel. Purification factors up to five and concentration factors of about eight could be achieved. The flux behaviour was analysed and interpreted according to existing models of filtration. The results were confirmed by scanning electron microscopy. The binding capacity of the membranes differed considerably with the mode of operation. The main component responsible for membrane fouling was identified by sodium dodecyl sulfate polyacrylamide gel electrophoresis and amino acid sequence analysis as bovine serum albumin or its fragments.

1. Introduction

Membrane chromatography is a new concept in downstream processing of proteins [1]. Membranes resemble the ideal form of a very short column [2] and possess the following advantages over conventional approaches in chromatography: high resolution according to their plate height values, [3] high flow-rates with low pressure drops according to their porosity [2,3], mainly convective transport of the biomolecules to the ligand [4], short cycle times, easy scaleability and high capacities caused by their large internal surfaces [5]. One important feature is that this technique allows a positioning of affinity methods in the early stages of downstream processing [6]. Depending on the type of ligand bound to the membrane they can be subdivided according to the interaction they exploit. For the purification of antibodies, filtration devices functionalized with protein A [7] or protein G [8] have been reported utilizing biospecific interactions. Aromatic amino acids [9] have been proposed as group-specific ligands. Other membrane adsorptions of monoclonal antibodies were performed with ion exchangers [10–12]. This technique exploits the electrostatic attraction between ligand and antibody and can be used with advantage only for antibodies with an isoelectric point sufficiently different from the bulk proteins, mainly albumin. Because of the large heterogeneity of immunoglobulins [13], this condition is not always met. In addition, the high ionic strength of culture supernatants may interfere with the ion-exchange process. To overcome this limitation, we concentrated our investigation on thiophilic ligands as a general method to capture antibodies [14]. This salt-promoted ad-

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sorption is reported to be highly selective for immunoglobulins and has been utilized for the purification of several classes of antibodies from different species [15–17]. Although the exact binding mechanism is unknown, one can regard this method as a group-specific affinity technique.

2. Experimental

2.1. Materials

Activated microfiltration membranes (Sartobind Epoxy) were obtained from Sartorius (Göttingen, Germany). These are nylon 66 membranes with a nominal pore diameter of 0.45 μm carrying epoxy groups. Divinyl sulfone was obtained from Sigma (Dreieich, Germany), sodium acetate, Tris and ammonium sulfate from Merck (Darmstadt, Germany) and acetone, methanol and diethyl ether from Fluka (Neu-Ulm, Germany). The molecular mass standards were taken from a Combithek kit from Boehringer (Mannheim, Germany). The PVDF membrane used for blotting was purchased from Millipore (Eschborn, Germany).

Hybridoma culture supernatant was generously provided by the cell culture group in the Institute of Biotechnology 2 (KFA Jülich, Jülich, Germany) where the cell line 425 from Merck was cultivated continuously in a fluidized bed reactor [18]. The antibody produced was an IgG_{2a}. Another cell line from Abion (Jülich, Germany) produced an IgG_1. The culture media contained 1% of foetal bovine serum and 100 mg/l of bovine serum albumin (BSA). The antibody concentration was approximately 20 mg/l.

2.2. Membrane modification

For the modification reaction, the general procedure described by Nopper et al. [19] was adapted. All steps described below were carried out at room temperature (20°C) in a well ventilated fumehood. Before coupling the Sartobind epoxy membranes were washed with 0.2 *M* Tris buffer (pH 8.0) for 0.5 h. For reductive cleavage of the epoxy groups the membranes were incubated with 0.3 *M* sodium hydrogensulfide in the same buffer for 2 h. After an extensive washing step with distilled water, acetone, methanol and diethyl ether for at least 3 h, the membranes were shaken in a solution containing 0.2 ml of divinyl sulfone per square centimetre of the apparent membrane surface in 0.2 *M* Tris buffer (pH 9) under a nitrogen atmosphere for 18 h, followed by washing steps as described above. Finally, an incubation with β-mercaptoethanol in Tris buffer (pH 8.5) was carried out for 18 h. After extensive shaking with washing reagents, the membranes were stored at 4°C in sodium acetate buffer (pH 5.2) containing 0.01% sodium azide.

The ligand density was calculated from elemental analyses (S, C) carried out in the Central Analytical Laboratory at the KFA Jülich. On the assumption of three sulfur atoms per ligand molecule, the ligand concentration was determined to 0.52 μmol per square centimetre of frontal membrane surface. To compare the membranes, adsorption experiments were performed with membranes lacking the thiophilic modification. It is known [20] that it is possible to immobilize proteins on epoxylated carriers at high ammonium sulfate concentrations, and we therefore reduced the epoxy groups with sodium hydrogensulfide and deactivated them with β-mercaptoethanol. These membranes are called "non-modified" in the following discussions.

2.3. Sample treatment

For dead-end experiments, solid ammonium sulfate was added slowly with stirring to 120 ml of cell culture supernatant until the final concentration was 1.0 *M*. The pH was adjusted to 5.2 with 25% HCl, then the sample was filtered through a 0.2 μm filter to remove any precipitated protein. For the experiments in the cross-flow mode the ammonium sulfate was first dis-

solved in equilibration buffer to a final concentration of 2.0 *M* and mixed with equal volumes of the supernatant. The pH was then adjusted to 5.2. The final sample had a volume of 9.0 l.

2.4. Dead-end filtrations

Six membrane discs with a diameter of 2.5 cm were fixed in small filtration modules of Plexiglas with inserted sintered stainless-steel plates [21] and equilibrated with 200 ml of 20 m*M* sodium acetate buffer (pH 5.2) containing 1.0 *M* ammonium sulfate. The pretreated samples were applied to the membranes with a Pharmacia P1 pump. The filtration device was then washed with 130 ml of equilibration buffer to remove non-adsorbed proteins. The protein concentration in the effluent was followed with a Uvicord I analytical UV monitor (Pharmacia). When the absorbance reached baseline levels the elution step was carried out using 20 m*M* Tris–HCl buffer (pH 8.0). From each step samples were collected and the protein concentration and the antibody content were determined as described below.

2.5. Cross flow filtrations

We examined the adsorption of monoclonal antibodies from hybridoma culture supernatants on the thiophilic membrane in a low-shear module with a spiral wound filtration channel [22]. The pretreated sample was pumped on to the membrane under recirculating conditions with a volumetric flow-rate of 80 l/h. This high flow-rate was necessary to give rise to a secondary stream also known as Dean vortices [23]. We employed an Ismatec (Zürich, Switzerland) gear pump with a Z-142 head. The protein concentration was observed with a Uvicord II preparative UV monitor (Pharmacia). The transmembrane pressure was kept constant at a level of $9.4 \cdot 10^4$ Pa and monitored by two Wilke 1 D precision manometers. The flux was analysed gravimetrically.

2.6. Determination of the filtration law in dead-end mode

To elucidate the filtration law for the dead-end mode of operation, membrane discs with a diameter of 5.0 cm were fixed with an ebonite ring on to the lower piston of a conventional chromatographic column. The pretreated hybridoma supernatant was added from the top before closing the column with a second piston. Nitrogen was applied to the column with a preset constant pressure of $1.3 \cdot 10^4$ Pa. The resulting flux was observed as a function of time by a precision balance. Analysis was carried out with a special computer program, FLUX (H. Schomberg, Institute of Enzyme Technology, Jülich), which allowed the raw data to be examined using established filtration laws [24].

2.7. Isolation of membrane fouling substances

For the identification of the substances fouling and clogging the membrane, at least 600 cm^2 of membrane was cut into small slices (0.5 mm × 1.0 cm) with a scalpel. The pieces were dropped into 1 l of 20 m*M* Tris buffer (pH 5.2) containing 0.01% sodium azide. After cutting the whole membrane sample (used in the dead-end or cross-flow mode), the pieces were transferred into 1 l of 20 m*M* glycine–HCl (pH 1.6) and shaken for 1 h. Finally, they were exposed to 12% HCl for 0.5 h. The acid solutions were neutralized by addition of solid Tris. Both fractions were freeze-dried and redissolved in 8 ml of Tris buffer. Buffer exchange against electrophoresis buffer [25] was carried out by gel permeation in PD 10 columns (Pharmacia). As the final concentration step, ultrafiltration was applied in columns with a cut-off of M_r 10 000, reducing the final volume to 0.07–0.3 ml.

2.8. Analytical methods

Sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) was carried out according to Laemmli [25] under denaturing and reducing conditions. The proteins were blotted from unstained gels on to the PVDF membranes

with a Multiphor II system (LKB). The blotting membranes were stained with amido black. Gels from which proteins were not transferred on to membranes were stained with Coomassie Brilliant Blue. Protein concentrations were determined by the method of Bradford [26]. The antibody concentration was measured on a Proanamabs analytical column (Biolytica, Lund, Sweden). A UV monitor could be used for detection because this column is able to separate the immunoglobulins from the other bulk proteins. Calibration was carried out with an IgG_{2a} standard of known concentration. The amino acid sequences were determined using a Model 477 A pulsed liquid sequenator connected with an on-line HPLC 120 A, System (Applied Biosystems, Weiterstadt, Germany). Sequence comparisons were made with the program Genepro 5.0 employing PIR 3.0 (September 1991). Scanning electron microscopy (SEM) was performed with a Leica S 300 instrument at the Institute für Schicht- und Ionenforschung, KFA Jülich.

3. Results and discussion

3.1. Breakthrough curves and antibody binding capacities in dead-end mode

To document the efficiency of the nylon membrane modification, we recorded the breakthrough profiles of total protein and IgG_{2a} on a stack of six thiophilic membranes and non-modified-membranes, respectively. As can be seen from Fig. 1, the modified membranes adsorbed the immunoglobulins completely from the first 25 ml applied. Elution of the bound antibody yielded a capacity of 17 $\mu g/cm^2$ of frontal membrane surface, while the non-modified membranes only retained 0.2 $\mu g/cm^2$. In addition to the antibody concentration during the frontal chromatography, the total protein concentration is plotted in Fig. 1. As can be seen, both modified and non-modified membranes show a similar breakthrough behaviour. Total protein breakthrough occurs almost instantaneously in both membranes, demonstrating that the selectivity of the thiophilic adsorption, as has been described by

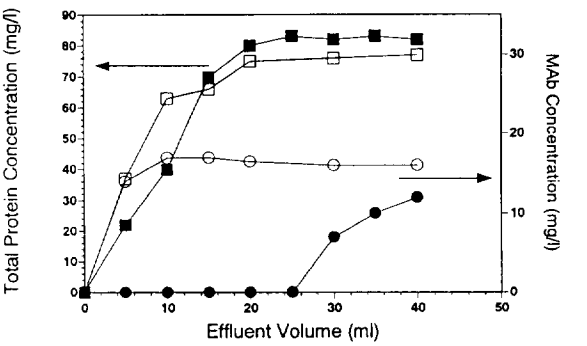

Fig. 1. Breakthrough profile of IgG_{2a} and total protein on a stack on six thiophilic membranes (closed symbols) and six non-modified membranes (open symbols). ● = IgG_{2a} concentration; ■ = total protein concentration. The filtration devices were equilibrated with 20 m*M* sodium acetate (pH 5.2) containing 1.0 *M* ammonium sulfate.

other groups for particulate adsorbents [14,19], is not changed by the transfer of this binding mechanism to membrane chromatography. The antibody is bound whereas most of the proteins pass through the membrane unretained. The applicability of the method was tested by examining the adsorption of an antibody of a different subclass on thiophilic membranes. Fig. 2 shows the breakthrough of total protein and IgG_1 on the same filtration device. In this instance the first 30 ml of the effluent fractions were free of antibody while again total protein breakthrough occurred almost instantaneously, showing the selectivity of the method. By eluting

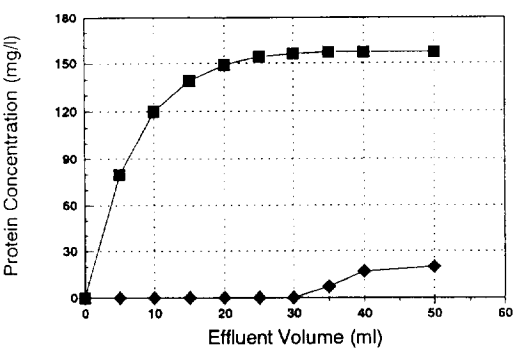

Fig. 2. Breakthrough profile of IgG_1 on a stack of six thiophilic membranes. ◆ = IgG_1 concentration; ■ = total protein concentration.

the bound antibody, the binding capacity for IgG_1 was determined as 16.3 $\mu g/cm^2$.

A point of major concern is the concentration of total protein in the breakthrough. The initial protein concentration in both supernatants (IgG_1 and IgG_{2a}) was 550 mg/l, whereas from Figs. 1 and 2 the breakthrough concentrations may be taken as 83 mg/l for the IgG_{2a} supernatant and 160 mg/l for the IgG_1 supernatant. Obviously the rest of the protein is adsorbed irreversibly on the membrane, a fact to which the restricted reusability of the membranes may be attributed (for details see below). It is important to note that in both instances the antibody concentration after breakthrough is identical with that in the feed, showing that the antibody is not irreversibly retained in the membrane. The irreversible protein adsorption is much less for the IgG_1 supernatant than the IgG_{2a}-containing solution. As the amount of antibody bound reversibly is similar, this means that the thiophilic filtration method is more specific for IgG_1 but the capacity, which is limited by ligand density, is fully exploited by binding an amount of 17 $\mu g/cm^2$ of frontal surface.

The purification and concentration of monoclonals by thiophilic membrane chromatography are summarized in Table 1. As discussed above, the purification of IgG_1 is more successful than that of IgG_{2a}, whereas the concentration factors achieved are identical. Different results were obtained with the same IgG subclass depending on the mode of filtration applied, which will be discussed below.

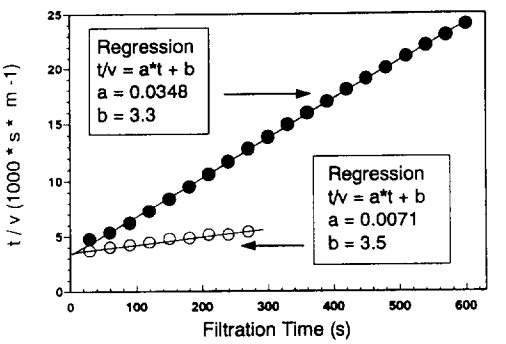

Fig. 3. Filtration law using (●) thiophilic and (○) non-modified membranes. The ratio of filtration time to specific filtrate volume is plotted against filtration time. The regression obtained had a correlation coefficient $r = 0.994$ (thiophilic membrane) and $r = 0.999$ (non-modified membrane).

3.2. Filtration law in dead-end mode

Fig. 3 shows the Hermans graphs of a modified and a non-modified nylon membrane. The ratio of filtration time to specific filtrate volume is plotted against the filtration time. A strictly linear dependence can be seen from the regressions. In both instances the filtration type obeys the standard filtration law, which implies that internal clogging occurs during filtration. Obviously, by this mechanism most of the proteins, which are not bound by the thiophilic mechanism, are irreversibly retained within the pores of the membranes leading to breakthrough of only a small amount of the proteins applied to the filtration device. This could be visualized by

Table 1
Results of the purification of monoclonal antibodies by thiophilic membrane chromatography

Mode of filtration	Antibody subclass	Antibody concentration in the eluate (mg/l)	Purification (-fold)	Concentration (-fold)
Dead end	IgG_{2a}	160	2.4	8
Cross-flow	IgG_{2a}	48	4.2	2.4
Dead end	IgG_1	176	5	8

Feed concentrations: IgG_1, MAb = 22 mg/l, total protein = 520 mg/l; IgG_{2a}, MAb = 20 mg/l, total protein = 500 mg/l.

Fig. 4. SEM of a cross section of a new thiophilic membrane.

SEM of membrane cross sections. Fig. 4 shows an unused nylon membrane and Fig. 5 indicates that the inner surface is coated with a protein layer after use. The proteins were identified as discussed below.

3.3. Comparison of cross-flow filtrations with dead-end experiments

Fig. 6 shows the flux profile of a filtration experiment in the spiral module. During the first hour the flux declines rapidly, then there is an asymptotic approach to a value of 0.19 l/h, corresponding to 18.7 $l/(m^2 \cdot h \cdot bar)$.

Fig. 5. SEM of a cross-section of a used thiophilic membrane.

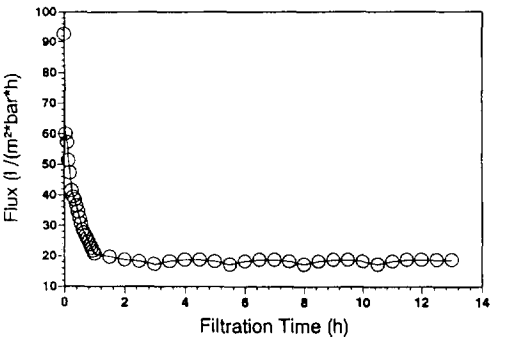

Fig. 6. Flux behaviour of a thiophilic membrane in the cross-flow mode. Conditions used: frontal membrane surface employed, 0.0108 m^2; preset pressure difference, $9.4 \cdot 10^4$ Pa; nominal pore size, 0.45 μm; feed flow-rate, 80 l/h.

Fig. 7 shows a balance of IgG_{2a} during cross-flow filtration. In this experiment a sample volume of 0.6 l was used and the ammonium sulfate concentration was adjusted by addition of solid salt to allow a comparison with the capacities in the dead-end mode. From this experiment an antibody binding capacity of 29 $\mu g/cm^2$ was calculated. This high value was not expected, because the membrane employed in cross-flow filtration was taken from the same modification lot as used for dead-end filtration. Additionally, an increased purification was achieved compared with the dead-end mode, as can be seen from Table 1.

To confirm this result, we performed a series of experiments determining the antibody adsorbed as a function of the amount of antibody

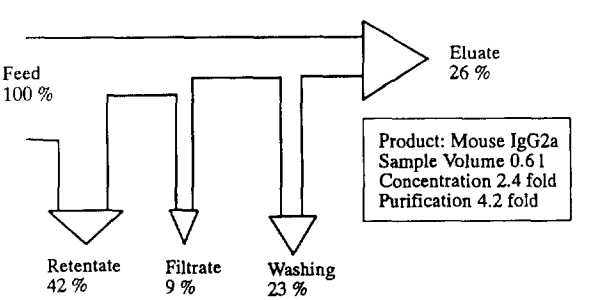

Fig. 7. Balance of IgG_{2a} in cross-flow mode under overload conditions.

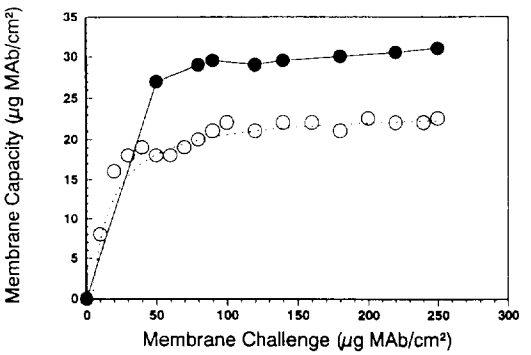

Fig. 8. Capacity data for (○) dead-end and (●) cross-flow mode of IgG_{2a} adsorption.

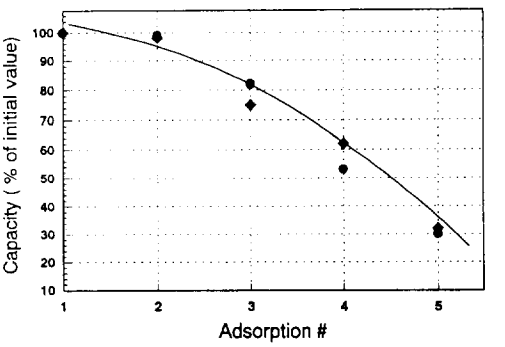

Fig. 9. Relative capacities of thiophilic membranes for IgG_{2a} binding at (◆) 1.0 *M* and (●) 0.75 *M* ammonium sulfate during repetitive runs.

in the sample employed per cm^2 of the filtration device (membrane load). Fig. 8 demonstrates the capacity differences between the two modes of operation. Whereas in the dead-end mode the maximum capacity was 20 $\mu g/cm^2$ of frontal membrane surface, in the cross-flow mode the capacity increased to 30 $\mu g/cm^2$. We tried to describe the flux behaviour of the cross-flow device with known models. The data could not be correlated by assuming either a standard filtration or a cake filtration, or an intermediate behaviour, as described by Hermans and Bredee [24]. Further work is needed to elucidate the underlying mechanism and the influence of the hydrodynamics in this special device used.

3.4. *Reusability of thiophilic membranes*

Generally, for dead-end filtrations of IgG_{2a}-containing supernatants on thiophilic nylon membranes, a membrane stack could be used only once. The clogging was so strong that it was difficult to pump any fluid through the stack. The common regeneration techniques using 0.1 *M* NaOH or 0.01% NaOH containing 20% 2-propanol failed to restore the water fluxes. When the filtrations were carried out with IgG_1-containing hybridoma supernatants, up to five experiments could be performed with a steady decline in capacity, as can be seen from Fig. 9. This may be attributed to the fact that the amount of unspecifically retained protein which does not appear in the breakthrough (see Figs. 1 and 2) is lower with IgG1. Obviously the internal clogging (see above) by this irreversible retention is limiting the reusability of thiophilic membranes.

The ammonium sulfate concentration (1.0 or 0.75 *M*) which is added to the feed to promote the thiophilic mechanism determines the absolute values for adsorption while the relative decline in capacity is identical at both salt concentrations, as shown in Fig. 9, and must be attributed to fouling.

In cross-flow experiments, the steady-state flux did not fall below 0.19 l/h, even after ten filtrations of 13 h duration. The initial flux velocity, however, diminished from run to run, as shown in Fig. 10.

We are currently working on the regeneration step in order to prolong the lifetime and reusability of the thiophilic membranes.

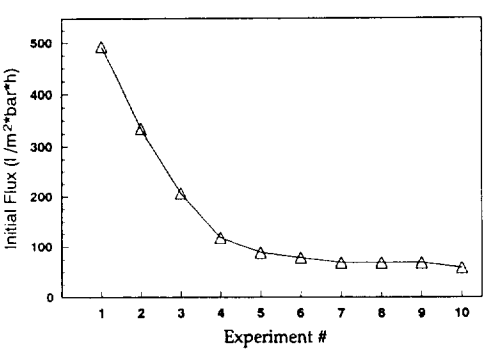

Fig. 10. Initial flux during ten cross-flow experiments.

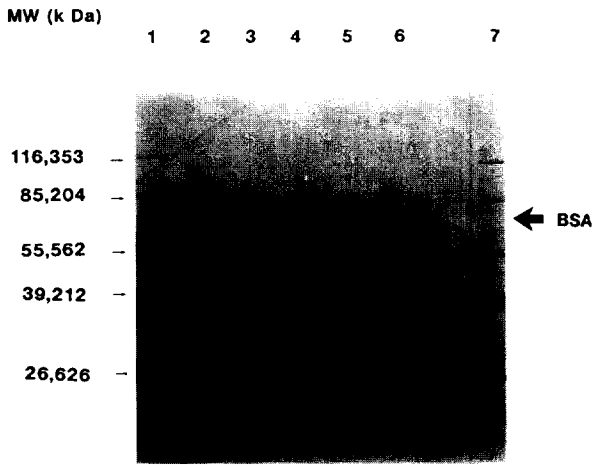

Fig. 11. SDS-PAGE (10%) of reduced samples with Coomassie Brilliant Blue staining. Lanes: 1 and 7 = molecular mass standards; 2 and 5 = proteins isolated from membranes employed in dead-end mode; 3 and 4 = proteins isolated from membranes used in the cross-flow mode; 6 = samples taken from the gel layer on the frontal surface of the membranes utilized in the cross-flow mode.

3.5. Identification of proteins responsible for internal clogging and membrane fouling

Fig. 11 depicts an electrophoretic profile of the substances isolated from the membranes. It clearly demonstrates that there is a difference in the nature of the proteins responsible for internal clogging dependant on the filtration mode. The proteins isolated from membranes employed in the dead-end mode are to be seen in lanes 2 and 5, while lanes 3 and 4 contain the substances isolated from membranes used in the spiral module in the cross-flow mode. During cross-flow filtration, a gel layer was formed on top of the membranes, and this layer was carefully taken off and recovered before cutting the membrane for further studies. Lane 6 represents the proteins contained in this layer after extensive washing and elution of the cross-flow module. Lanes 1 and 7 show the molecular mass standards. The sequence of the main band (M_r 67 000) in lanes 2 and 5 indicates that during dead-end filtration BSA is enriched in the membrane stack. After blotting, the first fifteen amino acids of the band were shown by Edman degradation to be identical with the known primary structure of BSA. BSA was found also in the gel layer on top of the cross-flow membrane, as can be seen from lane 6. The substances clogging the membrane during cross-flow filtration are not easy to identify, as the amounts are smaller and not all bands are homogeneous. For the band with M_r 24 000 (lanes 3 and 4), however, the sequence was identical with part of the albumin sequence starting from amino acid 381. One can state that fragments of that protein are enriched in the inner membrane pores during cross-flow operation, whereas whole BSA molecules are deposited in a gel layer on top of the membrane.

A point of concern during the cross-flow experiments was possible damage to proteins by the gear pump due to increased shear forces. The occurrence of BSA fragments in the pores of the membrane might suggest damage to proteins by the pump and subsequent deposition in the membrane pores. By performing SDS-PAGE of the retentate after 13 h of filtration we were able to exclude this assumption. Comparison of the protein pattern of the retentate with the pattern of the original feed solution shows no differences; the characteristic M_r 24 000 band of the BSA fragment is not found. Keeping in mind that the proteins in the retentate passed through the gear pump throughout the experiment (13 h), we conclude that the pump itself does not contribute to the occurrence of the BSA fragments and the protein damage takes place in the membrane pores during cross-flow filtration.

4. Conclusions

These experiments have shown that there are differences in the efficiency of thiophilic membranes to capture monoclonal antibodies by filtration of hybridoma culture supernatants. In part this is due to the antibody subclass employed. Another factor crucial for the capacity is the filtration mode. In dead-end filtrations a maximum capacity of 20 μg IgG_{2a}/cm^2 could be reached. During cross-flow filtration the maximum capacity as up to 30 $\mu g/cm^2$. The decline in flux during the filtrations is caused by different

mechanisms and substances depending on the filtration mode. During dead-end experiments internal clogging occurred, which can be deduced from the standard filtration law. The substance responsible for that is solely BSA. During cross-flow filtration a BSA gel layer builds up on top of the membrane and fragments of BSA are found in the inner pores. The hydrodynamics seem to influence the selectivity of the purification step by avoiding extensive internal clogging and masking of ligands. Our results partly confirm observations by Opong and Zydney [27] on the deposition of BSA on the frontal membrane surface. However, as can be seen from the data presented here, that is not sufficient to explain the total causes of the decline in flux during filtration. Filtration has to be regarded as a dynamic incident, as different quantities of diverse proteins or their fragments are deposited within the membranes depending on the filtration mode employed. This is supported by studies from Bowen and Gan [28] and Kim et al. [29], who found differences between protein layers adsorbed by diffusion of proteins when shaking the membranes in BSA solutions and protein deposition in flow-through conditions. For dead-end filtration, Bowen and Gan [30] described internal clogging of BSA within microfiltration membranes obeying the standard filtration law on filtering plain aqueous BSA solutions (0.1 g/l). The same behaviour was observed here employing cell culture supernatant (containing 0.1 g/l of BSA and 1.0 *M* ammonium sulfate) and either thiophilic or non-modified membranes. Therefore, this kind of fouling seems to be caused by specific properties of BSA. The concentration of BSA in cell culture media differs with cell lines, products and cultivation strategies [31]. Generally, an effort should be made to minimize the BSA concentration in order to improve the recovery of products with membrane processes as initial purification steps.

Acknowledgements

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